Utilization and metabolism of palmityl and oleoyl fatty acids and alcohols in caecal enterocytes of Atlantic salmon (*Salmo salar* L.)

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Keywords: Enterocytes; Fatty alcohol; Fatty acid; Uptake; Esterification; Oxidation; Metabolism; Wax ester; Atlantic salmon.

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Abbreviations: BHT, butylated hydroxytoluene; CO, copepod oil; FA, fatty acid; FAlc, fatty alcohol; FO, fish oil; HPTLC, high-performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol; TLC, thin-layer chromatography; WE, wax ester.

Abstract

The substitution of fish oil with wax ester-rich calanoid copepod-derived oil in diets for carnivorous fish, such as Atlantic salmon, has previously indicated a lower lipid digestibility. This suggests that the fatty alcohols (FAlc) present in wax esters may be a poorer substrate for intestinal enzymes than the fatty acids (FA) in triacylglycerol, the major lipid in fish oil. The hypothesis tested was that the possible lower utilization of dietary FAlc by salmon enterocytes is at the level of uptake and that subsequent intracellular metabolism was identical to that of FA. A dual-labelled FAlc-FA metabolism assay was employed to determine simultaneous FAlc and FA uptake and relative utilisation in enterocytes isolated from pyloric caeca of Atlantic salmon fed either a diet supplemented with fish oil or wax ester-rich *Calanus* oil. The diets were fed for 10 weeks before caecal enterocytes from each dietary group were isolated and incubated with equimolar mixtures of either [1-^{14}C]16:0 FA and [9,10(n)-^{3}H]16:0 FAlc, or [1-^{14}C]18:1n-9 FA and [9,10(n)-^{3}H]18:1n-9 FAlc. Uptake was measured after 2 h with relative utilization of labelled FAlc and FA calculated as a percentage of uptake. Differences in uptake were observed, with FA showing higher uptake than FAlc, and 18:1 chains a higher uptake than 16:0. A proportion of unesterified FAlc was possibly recovered in the cells, but the majority of FALc was recovered in lipid classes such as triacylglycerol and phospholipids indicating substantial conversion of FAlc to FA followed by esterification. However, incorporation of FA and FAlc into esterified lipids was higher when derived from FA than from FAlc. Twenty-five to fifty percentage of the absorbed 16:0 FAwas recovered in TAG fraction of the enterocytes compared with fifteen to seventy-five percentage of 18:1 FA. Twenty to thirty percentage of the absorbed 16:0 FA was recovered in the PC fraction of the enterocytes compared with only five to fifteen percentage of the 18:1 FA. Less than 15% of the fatty chains taken up by the cells was used for energy production, with significantly higher oxidation of 18:1 in enterocytes from fish fed the fish oil diet

compared to the *Calanus* oil diet. However, overall, dietary copepod oil had little effect on FAlc and FA metabolism. Metabolic modification by elongation and/or desaturation was generally low at 1-5% of uptake. We conclude that our hypothesis was generally proved in that the uptake of FAlc by salmon enterocytes was lower than the uptake of FA and that subsequent intracellular metabolism of FAlc was similar to that of FA. However, unesterified FAlc was possibly recovered in the cells suggesting that the conversion to FA may not be concomitant with uptake.

Introduction

The very long-chain, n-3 (or ω3) highly unsaturated fatty acids have important roles in human nutrition, reflecting their roles in critical physiological processes. The most important source of these essential nutrients in the human food basket is fish and, with declining fisheries, an increasing proportion is being provided by aquaculture. Marine copepods have the potential of being a novel source of n-3-rich marine lipid for use in feed for farmed fish (Olsen *et al*. 2004). The most abundant copepod species in the North Sea is *Calanus finmarchicus*. Like most calanoid copepods it stores lipid primarily as wax esters (WE) composed of a fatty acid (FA) esterified to a long-chain fatty alcohol (FAlc) (Sargent and Henderson 1986). Many fish, including juvenile Atlantic salmon, *Salmo salar* L., are known to feed on copepods containing substantial amount of wax esters (Rikardsen *et al*. 2004). It is therefore expected that the capacity to utilize WE should be well developed in fish from the marine environment.

The intestinal tract of salmonids has, as with many carnivorous marine fish, numerous of blind sacs termed pyloric caeca to aid digestion and absorption of diets naturally high in lipid. The caeca are the most important site of uptake of the products of lipid digestion including FA and FAlc (Olsen and Ringø 1997; Denstadli *et al*. 2004). These products have to be hydrolysed from their respective esters prior to uptake (Cowey and Sargent 1977), and WE

digestibility has been shown to be enhanced by increased amount of bile and lipases, and increased transit time in the pyloric caeca (Patton and Benson 1975; Tocher and Sargent 1984). Patton and Benson (1975) suggested that the pyloric caeca provide both increased exposure to lipases for hydrolysis and an increased surface area for absorption. However, WE are generally considered to be less accessible and poorly digested in mammals (Place 1992). Effects of substitution of triacylglycerol (TAG)-rich fish oil (FO) with WE-rich copepod oil (CO) in diets for Atlantic salmon on growth and nutrient utilisation has been investigated. Olsen *et al.* (2004) showed that smolts fed a 260 g kg^{-1} lipid diet containing 375 g kg^{-1} WE had similar growth and feed efficiency (500 g to 1500 g, FCR ~ 1.0) as fish maintained on FO alone suggesting good adaptation to the WE rich diet. These data suggested that, at this level of dietary WE, fish appear to be capable of adjusting the digestive capacity to maintain a high rate of utilisation. However, a recent unpublished study of Bogevik *et al*. showed that 250 g smolt fed a CO diet with almost 500 g kg^{-1} of the lipid (260 g kg^{-1}) as WE did not show the same capability to compensate for the high dietary WE level, as both growth and digestibility were lower in these fish compared to fish fed a FO diet. This suggested that there may be a upper limit for optimal utilization of dietary WE. Whether the reason is limited hydrolysis of WE or further limitation in conversion of FAlc to FA was unclear, but it is possible that the FAlc present in WE is a poorer substrate than FA for intestinal metabolism. There is therefore considerable interest in determining the pathways for the utilization and metabolism of FAlc in fish.

After intra-luminal hydrolysis and uptake into enterocytes, the products of lipid digestion can have different fates dependent on the competition between different pathways of metabolism (Henderson 1996). Specifically, recent studies have shown that appreciable metabolism of FA such as esterification, β-oxidation and elongation/desaturation occurs in caecal enterocytes isolated from salmonid fish (Tocher *et al*. 2002, 2004; Oxley *et al*. 2005).

Furthermore, early studies indicated that FAlc must be oxidised to the corresponding FA before they can be effectively utilized (Bauermeister and Sargent 1979). We reasoned that, in addition to digestion, differences in the utilization of WE and TAG may also involve differences between FA and FAlc in their uptake and metabolism by enterocytes. The hypothesis tested was that the possible lower utilization of dietary FAlc by salmon enterocytes is at the level of uptake into enterocytes and that subsequent intracellular metabolism was identical to that of FA. Thus, Atlantic salmon were fed one of two dietary treatments differing only in their lipid source, being either standard FO or calanoid CO. After 10 weeks of feeding, caecal enterocytes from each dietary group were isolated and incubated with equimolar mixtures of either [1-^{14}C]16:0 FA and [9,10(n)-^{3}H]16:0 FAlc, or [1-^{14}C]18:1n-9 FA and [9,10(n)-^{3}H]18:1n-9 FAlc. The specific objectives were to determine the relative utilization and metabolism of FA and FAlc including uptake into enterocytes, conversion of FAlc to FA, esterification into lipid classes, export of lipid classes, oxidation for energy production, and modification by elongation and/or desaturation.

Material and methods

Experimental animals, diets and sampling

One hundred Atlantic salmon smolts with a body mass of 86.7 ± 2.3 g were obtained from the University of Stirling Marine Environmental Research Facility (Machrihanish, Argyll, Scotland) and were randomly distributed into four 1.5 m tanks of 400-L capacity at the Institute of Aquaculture, University of Stirling, Scotland, at 25 fish per tank. The tanks were supplied with recirculated seawater (37 ppt) at a constant temperature of 10 ^{o}C at 400 L h^{-1} with additional air supplied through an air stone. They were subjected to a photoperiod regime of 12-h light:12-h dark. After acclimatization for 1 week, the fish were fed with two fishmeal-based diets of 450 g kg^{-1} protein and 260 g kg^{-1} lipid with the added oil (230 g kg^{-1})

being either FO or oil derived from calanoid copepods, *Calanus finmarchicus* (CO). Diet formulation and preparation was as described previously (Olsen *et al*. 2004) except for a higher content of WE in the CO. Thus, the lipid of the CO diet contained 480 g kg^{-1} WE (compared with 370 g kg^{-1} WE in Olsen *et al*. 2004), with TAG accounting for only 110 g kg^{-1} of total lipid. In contrast, the major lipid class of the FO diet was TAG, which accounted for more than 590 g kg^{-1} of the total lipid with no WE. The remaining lipid classes were generally similar in the two diets being mainly free fatty acid (FFA), phospholipid and cholesterol. The diets were fed to duplicate tanks of fish twice a day until satiation for 10 weeks. Prior to sampling, the fish were starved for 24 h to clear the intestinal tract. They were then anaesthetised in MS222 (Sigma-Aldrich, Poole, UK), and bulk weighed. Smolts fed the FO diet grew from 87 g to 183 g during the 71 day trial giving a daily specific growth rate (SGR) of 1.1. This was slightly lower than those maintained on the CO diet that grew from 87 g to 210 g with an SGR of 1.2, however, these growth differences were not significant. Six fish per tank were killed by a blow to the head, the intestinal tract removed, and pyloric caeca dissected and used for the preparation of enterocytes as described below.

Isolation of caecal enterocytes

Enterocyte suspensions were prepared using pyloric caeca pooled from two fish per tank resulting in six enterocyte preparations per dietary treatment. Isolated caecal enterocytes were prepared by collagenase treatment of chopped caecal tissue and sieving through a 100 μm nylon gauze with HBSS/Hepes essentially as described in detail previously (Tocher *et al*. 2002, 2004). The caecal cell preparations were predominantly enterocytes although some secretory cells were present. One hundred μl of the enterocyte suspensions were retained for protein determination according to the method of Lowry *et al*. (1951) after denaturation with 0.4 ml 0.25% (w/v) SDS/1 M NaOH for 45 min at 60 ^{o}C. The average protein concentration

of the enterocyte preparations was 3.5 ± 0.2 mg ml^{-1}. Previously, it was shown that > 90% of isolated trout caecal enterocytes remained viable after 2 h under the assay conditions (Tocher *et al*. 2004).

Preparation of labelled FAlc and substrate mixtures

Tritium-labelled 16:0 and 18:1n-9 FA were converted to the corresponding FAlc by $LiAlH_4$ reduction as described by Smith *et al.* (1993). In brief, 1 mCi 16:0 or 18:1 FA in 1.4 ml tetrahydrofuran (THF) was added dropwise into a solution of 300 mg $LiAlH_4$ in 5 ml THF, and the reaction stirred for 1 h at room temperature before the reaction was terminated by addition of 5 ml 10% H_2SO_4 to inactivate the remaining $LiAlH_4$. The mixture was extracted three times with diethyl ether and FAlc isolated and purified by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (60:40:2, by volume) as developing solvent. Two substrate mixes (C_{16} and C_{18}) were prepared each containing ^{14}C-labelled FA combined with the corresponding ^{3}H-labelled FAlc. Thus, the C_{16} mix contained [1-^{14}C]16:0 FA combined with [9,10(n)-^{3}H]16:0 FAlc and the C_{18} mix contained [1-^{14}C]18:1n-9 FA combined with [9,10(n)-^{3}H]18:1n-9 FAlc. Appropriate amounts of labelled and unlabelled FA and FAlc stock solutions were combined, solvent evaporated under oxygen-free nitrogen (OFN), and re-dissolved in pure ethanol so that the substrate mixes were equimolar for the FA and FAlc and contained the same concentration of radioactivity.

Incubation of enterocytes with labelled C16 and C18 mixes

Each pooled enterocyte suspension was distributed into two 25 cm^2 cell culture flasks (Nunclon, Nunc A/S, Denmark) in 4.5 ml aliquots and 100 µl of either the C_{16} or C_{18} FA-FAlc substrate mixes added so that the final FA and FAlc concentrations were both 25 µM, and 1 µCi of each isotope. After incubation for 2 h at 20 ºC, cells were harvested by gentle

resuspension and 0.5 ml withdrawn for estimation of β-oxidation as described below. The remaining 4 ml was centrifuged in a conical glass tube at 500 x *g* for 5 min. The supernatant was transferred to a clean test tube and 16 ml of ice-cold chloroform/methanol (2:1, v/v) added, and total lipid extracted according to Folch et al. (1957) and used for determining the export of FA and FAlc from the enterocytes after esterfication into lipid classes. The cell pellet was washed with 1% FA-free bovine serum albumin (BSA) in Hank's balanced salt solution (HBSS), centrifuged at 500 x g for 5 min, and re-suspended in 0.88% KCl. Fifty μl of the suspension was taken for determination of total uptake into the enterocytes. The cell suspension was then homogenized in 5 ml ice-cold chloroform/methanol (2:1, v/v) containing butylated hydroxytoluene (BHT) and the extracted lipid re-suspended in chloroform/methanol and divided into two equal portions for determination of incorporation into cellular lipids and elongation/desaturation as described below.

Determination of conversion of FAlc to FA and esterification into lipid classes

The incorporation of ^{14}C-labelled FA and ^{3}H-labelled FAlc into lipid classes was determined by single-dimension, double-development high-performance thin-layer chromatography (HPTLC). A portion of total lipid was applied to HPTLC plates as a 1 cm streak. Plates were developed to half-way in methyl acetate/isopropanol/chloroform/ methanol/0.25% aqueous KCl (25:25:25:10:9, v/v), and subsequently fully-developed in isohexane/diethyl ether/acetic acid (85:15:1, v/v) (Olsen and Henderson 1989). Lipid classes were visualized by exposure to iodine vapour and individually scraped into scintillation vials containing 2.5 ml scintillation fluid and radioactivity determined in a scintillation counter (United Technologies, Packard, UK). Results were corrected for counting efficiency and quenching of ^{14}C and ^{3}H.

Determination of enterocyte β-oxidation activities

Beta-oxidation was estimated by determining radioactivity in acid-soluble products by methods developed for rat hepatocytes (Frøyland *et al*. 1996; Madsen *et al*. 1998), and successfully applied to salmonid enterocytes (Tocher *et al*. 2002, 2004). The 0.5 ml of cell suspension withdrawn after incubation was homogenized (Ultra-Turrax T8/S8N-5g probe, IKA-Werke GmbH and Co., Slaufen, Germany), 100 µl of 6% FA free-BSA solution added, and protein precipitated by addition of 1 ml of ice-cold 4 M perchloric acid. Following centrifugation at 5000 x *g* for 10 min, 500 µl of the supernatant was transferred to a scintillation vial and radioactivity determined as above.

Determination of desaturation-elongation

Desaturation and elongation products in the cellular lipid were determined by argentation thin-layer chromatography (TLC) (Henderson and Tocher 1992). Fatty acid methyl esters (FAME) were prepared from cellular total lipids by acid-catalyzed transmethylation (Christie 2003). After addition of 2 ml of 2% $KHCO_3$, FAME were extracted twice with 5 ml of isohexane/diethyl ether (1:1, v/v) containing 0.01% BHT, solvent evaporated under OFN and resuspended in 100 µl isohexane (including BHT). TLC plates were impregnated with 2 g silver nitrate dissolved in 20 ml acetonitrile, and activated at 110°C for 30 min. FAME were applied as 2 cm streaks, and the plates fully developed in toluene/acetonitrile (95:5, v/v). Autoradiography was used to visualise the FAME by exposing the plate to Kodak MR2 film for 6 days. The areas of silica corresponding to individual FAME were scraped into scintillation vials, 2.5 ml of scintillation fluid added, and radioactivity determined as above.

Materials

[1-^{14}C] FA (50 mCi/mmol) and [9,10(n)-^{3}H] FA (30-60 Ci/mmol) were purchased from GE Healthcare UK Ltd. (Little Chalfont, UK). Anhydrous diethyl ether, BHT, HBSS, $LiAlH_4$,

collagenase (type IV), FA-free BSA, silver nitrate, perchloric acid and THF were obtained from Sigma-Aldrich Co. Ltd. (Poole, U.K.). HPTLC (10 x 10 cm x 0.15 mm) and TLC (20 cm x 20 cm x 0.25 mm) plates pre-coated with silica gel 60 were obtained from Merck (Darmstadt, Germany). Solvents were HPLC grade and were obtained from Fisher Scientific (Loughborough, UK).

Calculations and statistical analysis

Uptake of FA and FAlc was expressed in absolute terms (pmol mg^{-1} $protein^{-1}$) for the 2 h incubation period, whereas utilization of FA and FAlc in various metabolic pathways was expressed as a percentage of the total recovery (sum of radioactivity in cellular lipid classes, cellular unesterified FA or FAlc, total β-oxidation and exported lipid classes (excluding unesterified FA and FAlc)). Unless otherwise stated, all data are presented as mean ± S.D. (n=6) with each pooled cell suspension considered as n =1 for statistical analysis. Data reported as percentages were arcsine transformed before statistical analysis to ensure normal distribution. The significance of differences due to dietary treatment were determined by a Student´s t-test. As in almost all cases, there was no significant effect due to diet, the significance of differences due to substrates and diets were determined in fish fed FO by two-way ANOVA followed by the Tukey post hoc test. All statistical analyses were performed using STATISTICA 6.1 software (StatSoft Inc., Tulsa, USA). Differences were regarded as significant when $P < 0.05$ (Zar 1984).

Results

Uptake of [1-^{14}C] FA and [9,10(n)-^{3}H] FAlc into caecal enterocytes

Uptake into enterocytes was significantly higher for FA than for FAlc for both 16:0 and 18:1, and uptake of 18:1 was higher than that of 16:0 for both FA and FAlc (Fig. 1). Dietary

treatment had no significant effect on uptake of either FA or FAlc into caecal enterocytes. Thus, the highest uptake for both dietary groups was with 18:1 FA, at almost 0.5 pmol mg protein^{-1} over the 2-h period, which was around double the uptake of the fatty moiety that was taken up the least, 16:0 FAlc in enterocytes from salmon fed CO.

Incorporation and esterification into enterocyte cellular lipids

Irrespective of chain length, only a small proportion (< 5%; except 10% 16:0 FAlc) of the radioactivity from either the FA or FAlc was recovered unesterfied in enterocytes (Fig. 2A & B). The pool of intracellular unesterified FAlc was significantly higher when enterocytes were incubated with ^{3}H-16:0 FAlc than with ^{3}H-18:1 FAlc (Fig. 2B). There was no significant effect of dietary treatment on the recovery of radioactivity from either FA or FAlc in the intracellular unesterfied pools. These pools are termed "unesterified" rather than "free" as they are most likely protein-bound (Oxley *et al*. 2005).

The majority of radioactivity recovered in the cells was esterified into lipid classes. Generally, esterification into total lipids was greater for FA than for FAlc, but was unaffected by chain length or dietary treatment (Fig. 2C). The greatest proportions of intracellular radioactivity were esterified into TAG, with a tendency of lower FAlc compared to FA esterified into TAG and generally higher (non-significant) incorporation of C_{18} chains (45-60%) than for C_{16} chains (30-40%) (Fig. 3A). Twenty to thirty percent of the radiolabels were recovered in total phospholipids with almost 25% of C_{16} chains recovered in phosphatidylcholine (PC), while only one third of this was recovered in PC from enterocytes incubated with C_{18} chains (Fig. 3B). Recovery of radiolabelled FA and FAlc in the other phospholipid classes was lower, accounting for around 5%, 2% and 1% of the intracellular esterification in the case of phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), respectively (Fig. 3C-E). There was no significant difference

between FA and FAlc (at a specific chain length) or dietary treatment in esterification into phospholipid classes (Fig. 3).

Export in lipid classes from enterocytes

Export of lipids from the enterocytes was estimated by the proportion of radioactivity recovered in esterified extracellular lipid classes. Recovery of radiolabel in exported lipids was greatest for ^{3}H-18:1 FAlc, with around 10% of the total uptake being recovered in this fraction (Fig. 4A). This was significantly higher than recovery of radiolabel in extracellular lipid classes for enterocytes incubated with ^{14}C-16:0 FA, which was around 5% of total uptake. Dietary CO had no significant effect on export of lipids from the enterocytes. With all the labelled substrates, the majority of the radiolabel in extracellular lipids was recovered in TAG, with no significant differences between chain lengths or dietary treatments (Fig. 4B). Recovery of radiolabel in extracellular phospholipids was greatest with PC, followed by PE, with no clear effects of chain length or dietary treatment (Fig. 4CD).

β-oxidation

Enterocytes from fish fed the FO diet showed a significantly higher recovery of radiolabel in acid-soluble (fatty acid oxidation) products from the C_{18} substrates than enterocytes from fish fed the CO diet, with 10 – 13 % vs. 2 – 7 % of the radiolabel taken up being utilized as a substrate for β-oxidation (Fig. 5). In addition, recovery of radiolabel in oxidation products from C_{18} FAlc was significantly higher than from C_{18} FA in fish fed CO. There was no significant effect of dietary treatment or substrate (FA v. FAlc) on the oxidation of C_{16} chains.

Conversion by desaturation and/or elongation

Conversion of incorporated fatty chains by desaturation and/or elongation was significantly

higher for C_{18} chains, with approximately 5-6% of total uptake of 18:1 converted compared to around 1% of C_{16} chains (Fig. 6). However, elongation of 16:0 to 18:0 cannot be determined as they could not be separated in the analysis. The main products of 16:0 desaturation were 16:1 (38%) and 18:1 (62%), independent of substrate being FA or FAlc (Table 1). The products from 18:1 were 20:1 (37%) followed by 24:1 (29-32%) and 22:1 (16-21%). Recovery of FA and FAlc in 22:1 was higher in enterocytes from salmon fed FO, while elongation to 24:1 was higher in enterocytes from salmon fed CO (data not shown).

Discussion

Since bile-aided emulsification and transport of free FA is a physical process to a large extent, one would not expect to find a great difference between FA and FAlc in these processes. Although a recent study used albumin as a carrier for the delivery of FA to enterocytes from rainbow trout (Oxley *et al*. 2005), ethanol was the preferred mode of delivery in the present study as it would be independent of the nature of substrate, FA and FAlc. It is, of course, impossible to replicate the emulsification environment of the *in vivo* intestine and so any method of delivery has to be seen as an limitation to in vitro studies. Based solely on passive diffusion, uptake across the enterocyte membrane favours high lipophilicity and luminal fat concentrations (mM), while facilitated transport, via putative plasma membrane transporters, favours low luminal concentrations (μM) (Proulx *et al*. 1985; Carlier *et al*. 1991; Tso and Nauli 2004). The fact that uptake of FA was favoured over the less polar FAlc at the relatively low concentrations (25 μM) used in the present study may suggest that the cellular uptake was not exclusively dependent on passive diffusion. Indeed, mammalian research has shown there are many factors that determine cellular fat uptake including protein-meditated translocation across the membrane and cytoplasmic channelling toward specific metabolic pathway (McArthur *et al*. 1999), all of which are regulated by peroxisome proliferator-activated

receptors (PPAR) (Jump 2002). However, it is important to recognise the potential limitations of in vitro assays with the salmon enterocytes, as the normal in vivo architecture and cell polarity of the intestinal epithelial membrane may not be replicated. Thus, it is possible that increased uptake of FA over FAlc could be due to uptake on the nonluminal side of the enterocyte which is unlikely to have any exposure to or membrane transporters for FAlc in the in vivo situation.

Increasing uptake with increasing chain length (and decreased polarity) was previously shown in trout enterocytes (Perez *et al*. 1999; Oxley *et al*. 2005). In addition, monoenoic FA was digested better than saturated FA in Atlantic salmon, while the opposite was true for FAlc (Olsen *et al*. 2004). The present study showed uptake of 18:1n-9 > 16:0 for both FA and FAlc. However, the availability of free, unesterified FAlc in the intestinal lumen may also be a factor in vivo. The intra-luminal hydrolysis of TAG appears to be different to that of WE, which may result in FA being available in excess of FAlc in the intestine of fish fed WE (Olsen and Ringø 1997). Indeed, we observed in a recent study that, in salmon fed a diet containing almost 50% WE, the lipid in the hindgut was 36% WE, 15% free FA and 12% free FAlc (Bogevik *et al*., submitted). Although it is unclear how much free FAlc would be available in the pyloric caeca, there is a non-specific, bile salt-stimulated lipase with WE hydrolase activity that is sufficient for WE digestion in caeca (Olsen and Ringø 1997).

Current knowledge is relatively scarce with regard to the metabolism of absorbed FAlc in enterocytes. Previous studies have shown that FAlc are oxidized to FA and incorporated into TAG in several fish species (Patton and Benson 1975; Bauermeister and Sargent 1979), and Bauermeister and Sargent (1979) determined that the conversion of FAlc to FA occurred in the intestinal mucosa. The present study showed that FAlc was readily taken up into isolated enterocytes and was utilized in different metabolic pathways. Primarily, radioactivity from FAlc was recovered in esterified lipid classes indicating that the FAlc must have been predominantly converted to FA. However, a small amount of radiolabel from FAlc substrates

was recovered unesterified in the intracellular lipid, especially after incubation with 16:0 FAlc, which may indicate that there was a step-by-step conversion of FAlc to FA inside the cell, and that conversion to FAlc was not concomitant with uptake. However, although the cells was washed with 1% FA-free BSA in HBSS specifically to absorb extracellular FAlc, we cannot completely exclude the possibility that the unesterified FAlc recovered with the cellular lipids may be associated with the outside of the cell membrane rather than being intracellular. Both uptake and oxidation of FAlc to FA was not significantly enhanced in fish fed the CO diet indicating that there was no obvious adaptive response to a diet rich in FAlc. However, the pool of unesterified FAlc was smaller, and the pool of unesterified FA was larger, in enterocytes from fish fed the CO diet compared to fish fed FO, which may suggest more effective conversion of FAlc to FA in fish fed WE.

The above established that, once in the enterocytes, FAlc is mostly converted to FA. Thus, in the remaining discussion, when we talk about "FAlc" we are actually meaning the FA from FAlc after oxidation to FA. There appeared to be no difference between substrate in the incorporation of FA and FAlc into cellular lipid classes, with both predominantly incorporated into cellular TAG. Although relatively less FAlc than FA was incorporated into TAG, this was mainly due to the pool of unesterified FAlc which requires an extra step in the metabolism for the ^{3}H-substrate. Similarly, both C_{16} and C_{18} were mainly esterified into TAG (intracellular and exported), with generally higher 18:1 levels incorporated into TAG. This was consistent with 18:1n-9 usually being the most abundant FA in TAG body lipids (Henderson *et al.* 1982). The high incorporation of 18:1n-9 into TAG, as in the present study, suggests that either it was esterified in more positions than other FA, or esterified into more TAG molecules (Oxley *et al*. 2005). Esterification of FAlc and FA into phospholipids was similar, with C_{16} chains being preferentially incorporated into PC compared to C_{18} chains. This is consistent with PC generally showing the highest level of 16:0 compared to other

phospholipid classes (see Tocher 2003), and the long chain saturated FA requirement in PC for efficient chylomicron/VLDL formation that has earlier been well documented in both mammals and fish (Olsen *et al*. 1999, 2000, 2003).

The present study showed relatively rapid cellular metabolism of FA to different fates, with only 5% or less recovered in the unesterified pool irrespective of substrate or dietary treatment. The unesterified FA are presumably protein-bound to prevent cytotoxic effects, or present as intracellular mediators of lipid metabolism through targeting specific enzymes or protein receptors including transcription factors (Storch and Thumser 2000). In addition less than 20% of the dual-labelled substrate taken up was used for energy production. Perhaps surprisingly, this was the only pathway that was significantly affected by dietary treatment with less 18:1 FA and FAlc substrate oxidised in fish fed CO compared to fish fed FO. Previous studies have shown effective utilisation of CO in salmonid fish with the assumption that 20:1 and 22:1 FA, derived from the long-chain monoenoic FAlc, are an important source of energy (Bauermeister and Sargent 1979; Sargent *et al*. 1979; Tocher 2003; Olsen *et al*. 2004). However in trout enterocytes, β-oxidation of 20:1n-9 FA was relatively low and it was postulated that FA oxidation in caecal enterocytes is not a simple function of dietary FA abundance (Oxley *et al*. 2005). In the present study, there was a trend, significant for 18:1, of higher oxidation of FAlc than FA, and this may be related to the continuous conversion of more FA from FAlc, subsequently available for different metabolic fates.

The final metabolic pathway investigated was further elongation and/or desaturation of the incorporated fatty chains. It is clear that this pathway was not a major fate for the saturated and monounsaturated C_{16} and C_{18} chains in this *in vitro* system. Once again, the recovery of radioactivity in the products of this pathway was identical for both initial sources of radioactivity, FA or FAlc, and no effect of diet was observed. No desaturation of 18:1 was observed, but elongation up to 24:1 was found. This is entirely expected as desaturase activity

will be repressed in fish fed diets replete with long-chain n-3 FA (Tocher 2003). The data appear to show that metabolism by elongation was greater for 18:1 than 16:0, but this is an artifact of the assay. Unfortunately, the separation of 16:0 and 18:0 by argentation TLC is not possible and so the amount of 16:0 that was chain elongated and not subsequently desaturated cannot be determined. However, 16:0 was elongated as the majority of desaturated products from 16:0 were 18:1 rather than 16:1. Interestingly, 18:1 was the limit of elongation of 16:0, compared to 24:1 with 18:1. The reason for this difference is unclear, but increased incubation time may result in further elongation.

In conclusion, we employed a dual-labelled assay to determine the simultaneous uptake and relative utilization of FAlc and FA in enterocytes isolated from pyloric caeca of Atlantic salmon fed diets supplemented with either FO or WE-rich CO. The hypothesis tested was generally proved in that the uptake of FAlc by salmon enterocytes was lower than the uptake of FA, in this in vitro model at least, and that subsequent intracellular metabolism of FAlc was similar to that of FA. However, unesterified FAlc was possibly recovered in the cells indicating that the conversion to FA may not be concomitant with uptake. The lower uptake, along with previously observed differences in digestion, may explain the possible lower utilization of dietary FAlc by salmon. The general lack of effect of the CO diet suggested little adaptation of enterocyte metabolism to high dietary FAlc content. Alternatively though, the lack of dietary effects was also evidence of no obvious physiologically detrimental effects of feeding a WE-rich diet utilizing oil derived from calanoid copepods.

Acknowledgement

This work was supported by the Norwegian Research Council (Grant no. 165051/S40). We would like to thank staff at the Institute of Aquaculture, University of Stirling, Robert Aitken

for fish husbandry, and Dr. R.J. Henderson and J.R. Dick for assistance and technical expertise.

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Figures legends

Fig. 1. Total uptake of individual [1-^{14}C]-labelled fatty acids (FA) and [9,10(n)-^{3}H]-labelled fatty alcohols (FAlc) by caecal enterocytes isolated from Atlantic salmon fed diets containing either fish oil (FO) or *Calanus* oil (CO). Columns represent the means of 6 replicates ± S.D. Values not having the same superscript letter are significantly different ($P < 0.05$) as determined by two-way ANOVA followed by the Tukey post hoc test for substrates.

Fig. 2. Recovery of radioactivity from [1-^{14}C]-labelled fatty acids (FA) and [9,10(n)-^{3}H]-labelled fatty alcohols (FAlc) in different lipid fractions of enterocytes from Atlantic salmon fed diets containing either fish oil (FO) or *Calanus* oil (CO). A, unesterified FA; B, unesterified FAlc; C, esterified lipids. Columns represent the means of 6 replicates ± S.D. Values not having the same superscript letter are significantly different ($P < 0.05$) as determined by two-way ANOVA followed by the Tukey post hoc test for substrates.

Fig. 3. Esterification of [1-^{14}C]-labelled fatty acid (FA) and [9,10(n)-^{3}H]-labelled fatty alcohol (FAlc) into individual lipid classes of isolated enterocytes from Atlantic salmon fed diets containing either fish oil (FO) or *Calanus* oil (CO). A, triacylglycerol (TAG); B, phosphatidylcholine (PC); C, phosphatidylethanolamine (PE); D, phosphatidylserine (PS); and E, phosphatidylinositol (PI). Columns represent the means of 6 replicates ± S.D. Values not having the same superscript letter are significantly different ($P < 0.05$) as determined by two-way ANOVA followed by the Tukey post hoc test for substrates.

Fig. 4. Recovery of radioactivity from [1-^{14}C]-labelled fatty acids (FA) and [9,10(n)-^{3}H]-labelled fatty alcohols (FAlc) in esterified lipid classes isolated from the extracellular medium of enterocytes from Atlantic salmon fed diet containing either fish oil (FO) or *Calanus* oil (CO). A, Total esterified lipids; B, triacylglycerol (TAG); C, phosphatidylcholine (PC) and D, phosphatidylethanolamine (PE). Columns represent the means of 6 replicates ±S.D., values not having the same superscript letter are significantly different ($P < 0.05$) as determined by two-way ANOVA followed by the Tukey post hoc test for substrates.

Fig. 5. Recovery of radioactivity from [1-^{14}C]-labelled fatty acids (FA) and [9,10(n)-^{3}H]-labelled fatty alcohols (FAlc) in the acid-soluble fraction of enterocytes from Atlantic salmon fed diet containing either fish oil (FO) or *Calanus* oil (CO). Columns represent the means of 6 replicates ±S.D., values not having the same superscript letter are significantly different ($P < 0.05$) as determined by two-way ANOVA followed by the Tukey post hoc test for substrates.

Fig. 6. Recovery of radioactivity from [1-^{14}C]-labelled fatty acid (FA) and [9,10(n)-^{3}H]-labelled fatty alcohol (FAlc) as desaturated and elongated products in enterocytes from Atlantic salmon fed diets containing either fish oil (FO) or *Calanus* oil (CO). Columns represent the mean of 6 replicates ± S.D. Values not having the same superscript letter are significantly different ($P<0.05$) as determined by two-way ANOVA followed by the Tukey post hoc test for substrates.

Tables

Table 1. Products of metabolic conversions of the radiolabelled fatty acids and fatty alcohols as percentage of the radioactivity recovered in caecal enterocytes from Atlantic salmon fed a fish oil diet.

Products	[1-^{14}C]-fatty acid substrate		[9,10(n)-^{3}H]-fatty alcohol substrate	
	16:0[a]	18:01	16:00	18:01
SAT		1.13±0.51		0.80±0.30
16:1 n-7	0.40±0.08		0.28±0.05	
18:1 n-9	0.65±0.12		0.46±0.09	
20:1		2.81±1.39		2.36±1.10
22:1		1.22±0.51		1.33±0.51
24:1		2.44±1.26		1.89±0.90

[a] A major product of 16:0 metabolism will be conversion by elongation to 18:0 but this cannot be separated from 16:0 by the solvent system used and cannot be included in the analyses.

Figure 1.

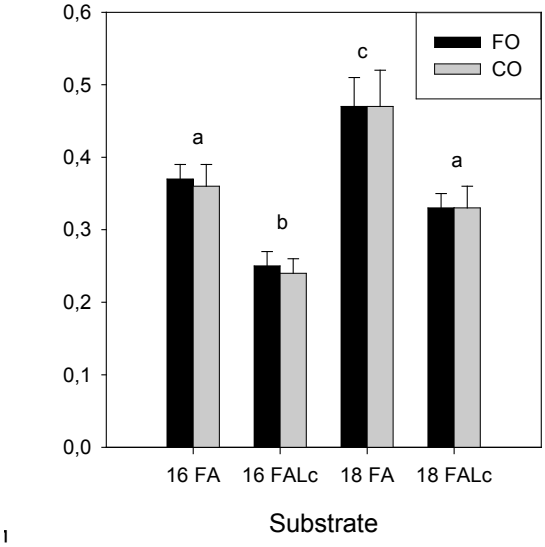
0,6
0,5
0,4
0,3
0,2
0,1
0,0
FO
CO
a
b
c
a
16 FA
16 FALc
18 FA
18 FALc
Substrate

Figure 2.

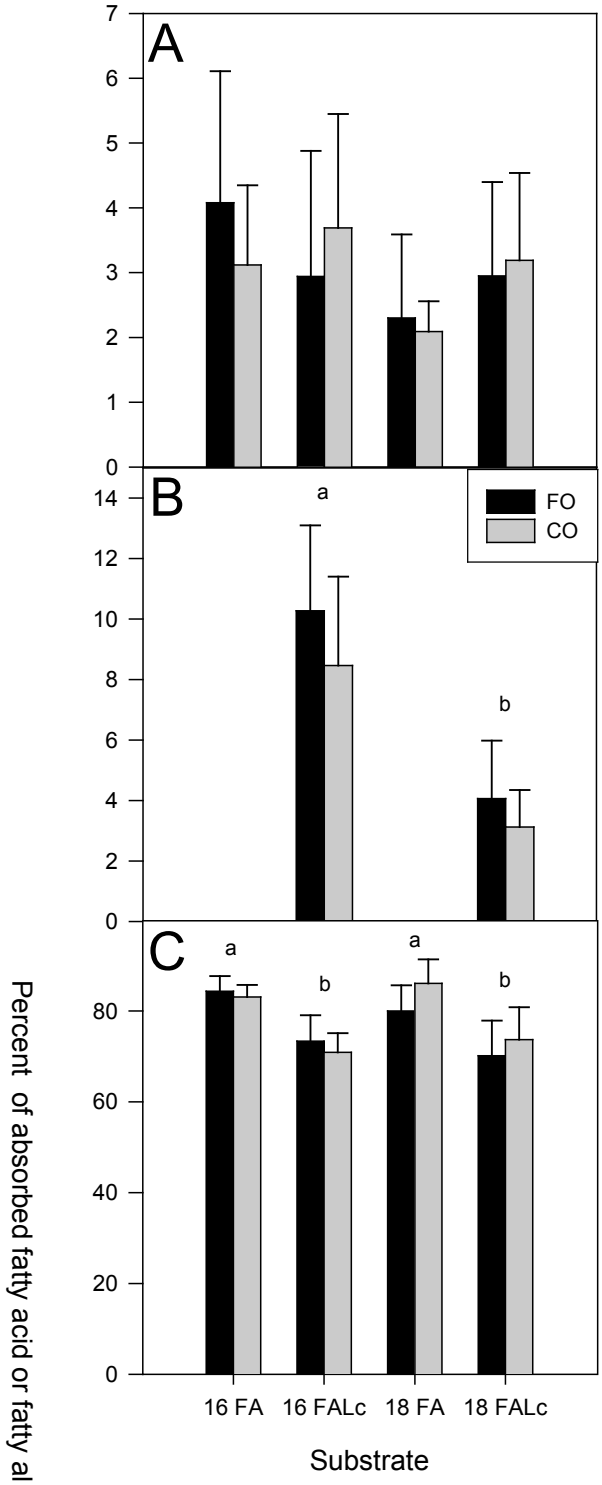
A
B
C
FO
CO
a
b
a
b
a
b
16 FA
16 FALc
18 FA
18 FALc
Substrate
Percent of absorbed fatty acid or fatty al

Figure 3.

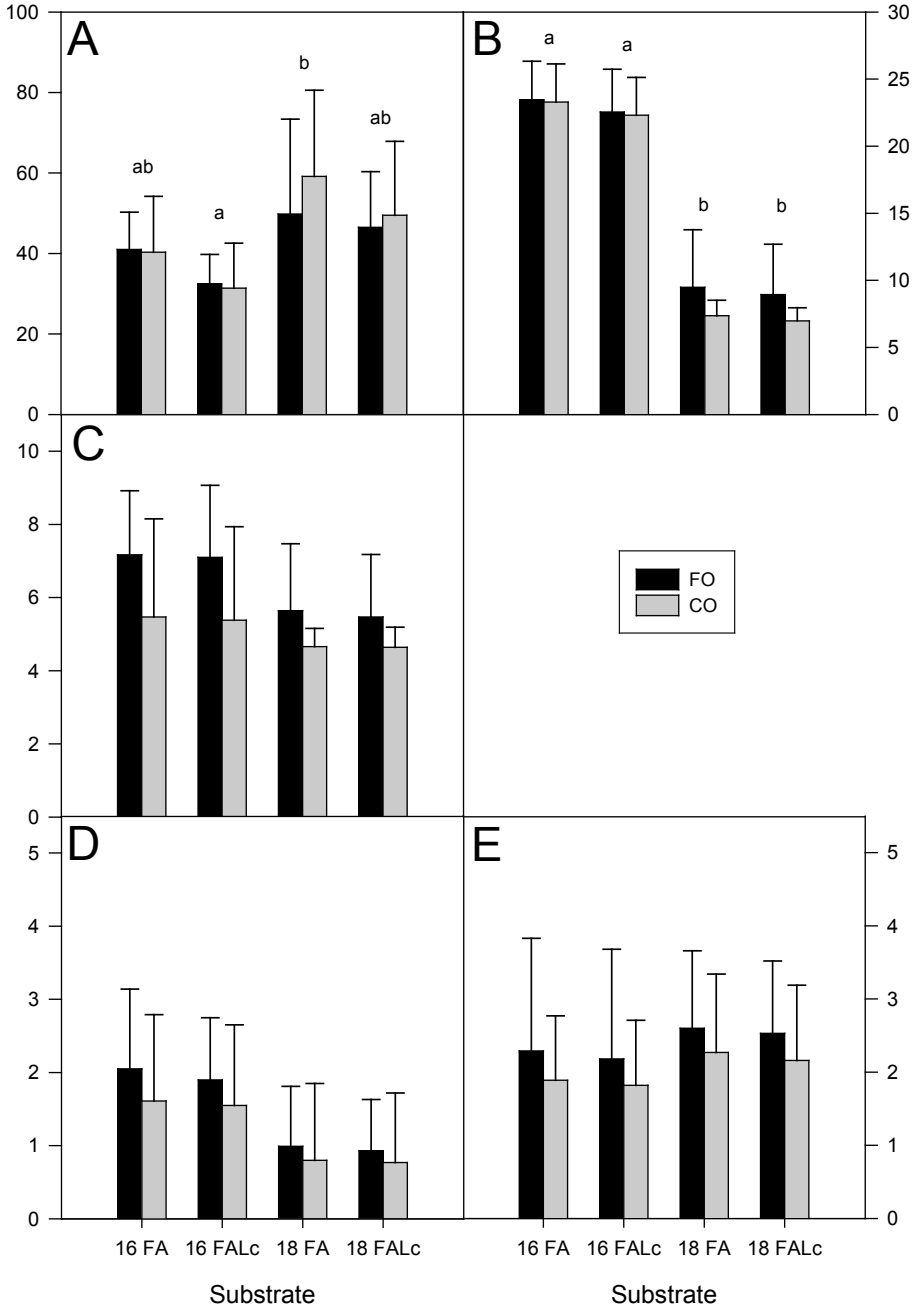

Figure 4.

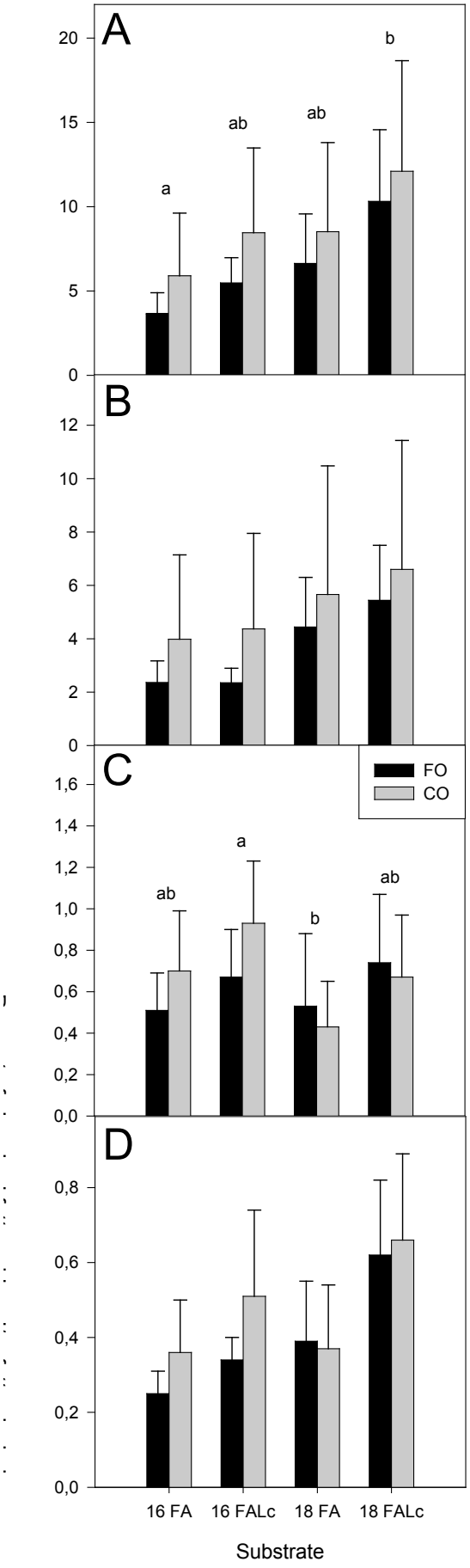
A
B
C
D
FO
CO
a
ab
ab
b
ab
a
b
ab
16 FA
16 FALc
18 FA
18 FALc
Substrate

Figure 5.

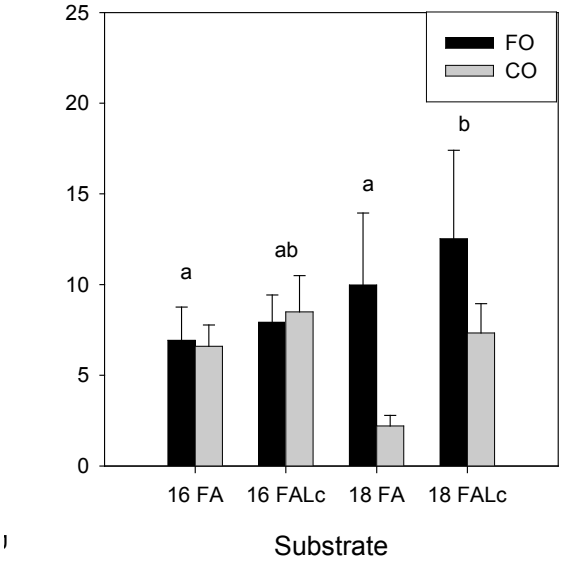

Figure 6.